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Acute reduction of TxA_2 platelet binding sites after *in vivo* administration of a TxA_2 receptor inhibitor

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1 Picotamide has been shown to interfere competitively with the thromboxane A_2 (TxA_2) platelet receptor. In the present study the effect of *in vivo* administration of picotamide on TxA_2 human platelet receptors was investigated in 10 healthy subjects.
2 Picotamide (300 mg × 3 daily) or placebo were administered in a double-blind, cross-over, placebo controlled study, each treatment lasting 1 week with a 2 week interval period. TxA_2 receptors were investigated by a direct radioligand binding assay method employing [^{125}I]-PTA-OH as labelled ligand. Platelet studies were performed on the first day of treatment immediately before and 2, 4 and 8 h after the ingestion of the drug. The effects of chronic administration were assessed on the seventh day.
3 Two and 4 h after the administration of picotamide 300 mg orally platelet TxA_2 receptors were significantly reduced from 1366 ± 237 to 957 ± 221 ($P < 0.05$) and 753 ± 119 receptors/platelet (mean ± s.d.) ($P < 0.03$). After 8 h platelet receptor population was restored (1362 ± 324, NS). The same pattern was observed after 7 days of treatment. Thus picotamide seems to induce a short lasting down regulation of platelet TxA_2 receptors.

Keywords picotamide thromboxane A_2 platelets

Introduction

The exposure of cells to agonists often results in a short or medium term decrease in responsiveness of the cells to the agonist (Benovic *et al.*, 1985; Harden, 1983), frequently accompanied by a loss in the specific membrane receptors for the agonist. This agonist-induced desensitization has also been reported for platelets. In fact anti-platelet drugs with specific agonist effects on PGI_2 or PGD_2 receptors have been reported to induce down-regulation of specific membrane platelet receptors either after *in vitro* incubation (Alt *et al.*, 1986; Jaschonek *et al.*, 1988) or *in vivo* administration (Modesti *et al.*, 1987) and this phenomenon limits the efficacy and the usefulness of these drugs. This down-regulation pattern was also observed to occur *in vitro* after platelet incubation with a TxA_2 analogue (Crouch & Lapetina, 1989; Liel *et al.*, 1988; Murray & FitzGerald, 1989). The reduction in TxA_2 receptor number *in vivo* after the administration of TxA_2 inhibitors might represent a useful additional effect for drugs interacting with specific TxA_2 receptors. This study was undertaken to investigate whether the administration of a drug with TxA_2 receptor antagonist activity (picotamide) to healthy subjects induced changes in affinity and/or density of the TxA_2 platelet receptors, assessed by the binding of the labelled antagonist [^{125}I]-PTA-OH.

Methods

Materials

ONO11120 (9,11-dimethylmethano-11,12-methane-16-phenyl-13,14-dihydro-13-aza-15-tetranor-TxA_2) (Narumiya *et al.*, 1986) was a kind gift of Dr Narumiya (Kyoto, Japan). [^{125}I]-PTA-OH (2000 Ci mmol^{-1}) (9,11-dimethylmethano-11,12-methano-16-(3 [^{123}I]-4-hydroxyphenyl)-13,14-dihydro-13-aza-15-tetranor-TxA_2), the labelled hydroxylated form of ONO11120, was obtained from Amersham (Buckinghamshire, GB). Prostacyclin (PGI_2) was obtained from Upjohn (Kalamazoo, Mi, USA). Picotamide (*N*,N'bis(3-picolyl)-4-methoxy-isophthalamide) (batch no 870311) was provided by the SAMIL inc.-Sandoz Group, Rome, Italy. All the other reagents were obtained from Merck (Darmstadt, F.R.G.) and were of analytical grade.

Experimental procedure

Ten healthy subjects aged 44 to 62 years were treated with placebo or picotamide (Plactidil, Samil-Sandoz, Italy) 300 mg three times daily orally in a double-blind, cross-over placebo controlled clinical study. The two treatments, each lasting 1 week, were separated by a 1

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week wash-out interval. No subject had taken any other drug for at least 15 days preceding the study. Blood samples for binding studies were collected on the first and on the last day of each treatment period before the first administration (08.00 h) of picotamide or placebo respectively and 2, 4 and 8 h after picotamide or placebo administration.

Blood sampling and platelet isolation

Blood was withdrawn by clean venepuncture and anti-coagulated with 15% (v/v) acid citrate dextrose (NIH formula A).

Platelet-rich plasma was prepared as previously described (Neri Serneri *et al.*, 1984). Platelet counts were performed by an automatic counter (Platelet analyzer 810, Baker Instruments, Allentown, PA, USA). Platelet-rich plasma, adjusted to pH 6.5 with acid citric dextrose, was centrifuged at 1,800 *g* for 30 min at room temperature (20–22° C). Platelets were then resuspended in 10 ml of phosphate buffer pH 7.2 (8 mmol l^{-1} Na_2HPO_4, 2 mmol l^{-1} NaH_2PO_4, 10 mmol l^{-1} KCl, 135 mmol l^{-1} NaCl) and again recentrifuged at 1,800 *g* for 30 min at room temperature. The supernatant was discarded and the platelets were resuspended in assay buffer containing 138 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA and 25 mmol l^{-1} Tris/HCl, pH 7.5. If necessary assay buffer was added to obtain a platelet concentration of 10^9 platelets ml^{-1}.

Binding studies

Kinetics of ^{125}I-PTA-OH binding The binding of 0.5 nmol l^{-1} [^{125}I]-PTA-OH (final concentration, f.c.) to 10^8 platelets at room temperature, in a final volume of 0.2 ml was preliminarily evaluated at selected time intervals Non-specific binding was assessed at each time by adding 0.02 ml of ONO11120 (2×10^{-5} mol l^{-1}, f.c.). Specific binding at each time was then calculated as the difference between total and non-specific platelet bound radioactivity. After 10 min the reversibility of the binding was assessed by adding a large amount of unlabelled ligand (0.02 ml of ONO11120, 2×10^{-5} mol l^{-1}, f.c.) to the reaction mixture and the residual binding was evaluated at selected time intervals.

[^{125}I]-PTA-OH binding isotherms Platelet suspension (0.1 ml) was incubated in a final volume of 0.2 ml with [^{125}I]-PTA-OH 0.05 nmol l^{-1} (f.c.) (2000 Ci $mmol^{-1}$), plus ONO11120 at increasing selected concentrations (0 to 4×10^{-6} mol l^{-1}) for 10 min at room temperature. The residual radioactivity after the addition of ONO11120 2×10^{-5} mol l^{-1} (f.c.) was considered as non-specific binding. Non-specific binding of 0.05 nmol l^{-1} [^{125}I]-PTA-OH amounted to 25–35% of total bound radioactivity.

After 10 min incubation four 4 ml aliquots of ice-cold buffer were added to each tube to stop the reaction and the contents were rapidly filtered under reduced pressure through Whatman GF/C glass microfiber filters. Previous time course experiments showed that under these conditions binding equilibrium had been reached. The entire washing procedure was completed within about 15 s. Filters were dried under air flow and counted in a Beckman gamma counter with an overall efficiency of 50%. The experiments were carried out in triplicate.

Analysis of data

Kinetic studies The kinetic constants were calculated by time course experiments according to Weiland & Molinoff (1981). Briefly, the observed association constant (K_{obs}) was calculated as the slope of the pseudo-first order plot:

$$\ln(\text{LReq})/((\text{LReq}) - (\text{LRt})) \textit{ vs } \text{time } (t).$$

LReq and LRt respectively indicate the concentration of ligand-receptor complex at equilibrium and at each time (t).

The dissociation rate constant K_{-1} is a first order rate constant because the rate is determined after elimination of the forward reaction following prior incubation of ligand and receptor. The first order integrated rate equation for dissociation is:

$$\ln(\text{LRt})/(\text{LR}) = -K_{-1t}$$

LR and LRt are the concentrations of ligand-receptor complex just prior to the addition of competing ligand (time 0) and at time t respectively. The association constant (K_{+1}) was then calculated by the equation:

$$K_{+1} = (K_{ob} - K_{-1})/[\text{L}]$$

The kinetically determined dissociation constant (K_d) is finally given by:

$$K_d = K_{-1}/K_{+1}$$

Binding isotherms The total binding for each concentration in displacement curve at equilibrium was determined by dividing the decay per minute (d min^{-1}) of each platelet pellet by the calculated specific activity in d min^{-1} mol^{-1} (Modesti *et al.*, 1985). The analysis of these binding isotherms was performed according to Scatchard (1949).

Hill Plot of the displacement curve and K_i were calculated according to Cheng & Prusoff (1973).

Statistical analysis

If not otherwise indicated, all data given in the text are mean ± 1 s.d. of n experiments. The receptor numbers and the dissociation constants at each time were compared by analysis of variance.

Results

Time course and binding isotherms of the [^{125}I]-PTA-OH binding

[^{125}I]-PTA-OH rapidly bound to intact human platelets (Figure 1). The observed rate constant (K_{obs}) for specific binding was 0.340 min^{-1} (the equation for the line was $y = -0.026 + 0.340x$; $n = 5$). The dissociation curve

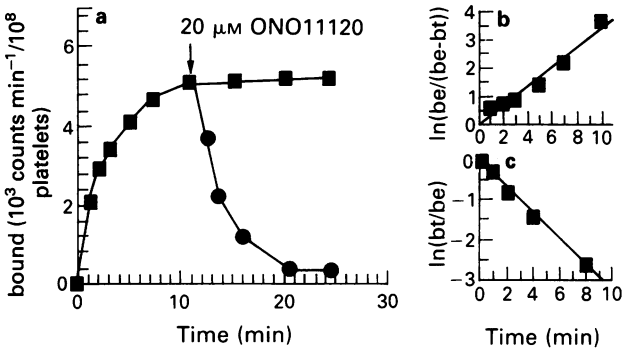

Figure 1 a) Time course of the association and dissociation phase of the binding of [^{125}I]-PTA-OH (0.5 nmol l^{-1}) to washed human platelets at room temperature. Dissociation was obtained after 10 min incubation upon addition of 20,000 nmol l^{-1} ONO11120 to the incubation mixture. Data points represent means of triplicate determinations in at least five independent experiments. b) Specific binding association is plotted according to the pseudo-first order rate equation (K_{obs} = 0.340 min^{-1}). c) Dissociation is plotted as a first order reaction (K_{-1} = 0.324 min^{-1}; $K_1 = (K_{obs} - K_{-1})\,1^{-1}$ = 0.032 nmol l^{-1} min^{-1}; $K_d = K_{-1}/K_1$ = 10 nmol l^{-1}).

performed after 10 min of incubation showed a rapid displacement of about 65–75% of the total radioactivity bound. The analysis of the first order rate of dissociation showed a linear pattern with a K_{-1} of 0.324 min^{-1} (n = 5). The association rate constant (K_1) was 0.032 nmol l^{-1} min^{-1} and the resulting kinetically determined dissociation constant K_d was 10 nmol l^{-1}.

The saturation of [^{125}I]-PTA-OH binding occurred at a ligand concentration about 100 nmol l^{-1} (Figure 2). The Scatchard analysis of this binding yielded a straight line, indicating a single class of binding sites for [^{125}I]-PTA-OH (Figure 2). The equilibrium dissociation constant (K_d) and the maximum concentration of binding sites (B_{max}) determined by Scatchard analysis, gave values of 21.5 ± 7.2 nmol l^{-1} and 198 ± 63 fmol/10^8 platelets (1194 ± 379 binding sites per platelet, n = 12) respectively. Hill coefficient (n Hill) of the binding was 0.942 ± 0.099, suggesting the existence of homogeneous individual class of binding sites without cooperativity (Figure 3).

Effect of picotamide administration The number or affinity of TxA_2 receptors did not undergo any significant changes after the administration of placebo. In contrast, a significant reduction of platelet membrane receptors was found 2 h (957 ± 221 receptors/platelet, $P < 0.05$) and 4 h (753 ± 119 receptors/platelet, $P < 0.03$) after picotamide administration in comparison with placebo administration. After 8 h platelet receptor population was completely restored (1362 ± 324 receptors/platelet, NS) (Figure 4) (Table 6). No changes in the affinity for the ligand were observed after picotamide.

The chronic administration of the drug for 1 week did not induce a significant change in TxA_2 platelet receptors. In fact binding studies did not show any significant differences in patients treated with picotamide when compared with patients treated with placebo before drug administration.

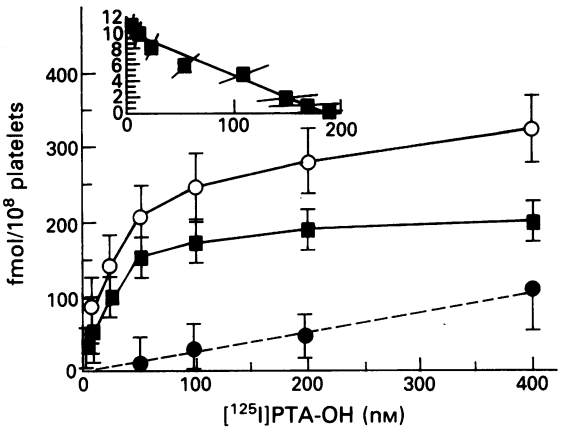

Figure 2 Saturation curve of the [^{125}I]-PTA-OH binding to washed human platelets at room temperature. (○ = total binding; ■ = specific binding; ● = non specific binding) (n = 12). Inset: Scatchard analysis of the specific binding (B_{max} = 198 ± 63 fmol/10^8 platelets, K_d = 21.5 ± 7.2 nmol l^{-1}).

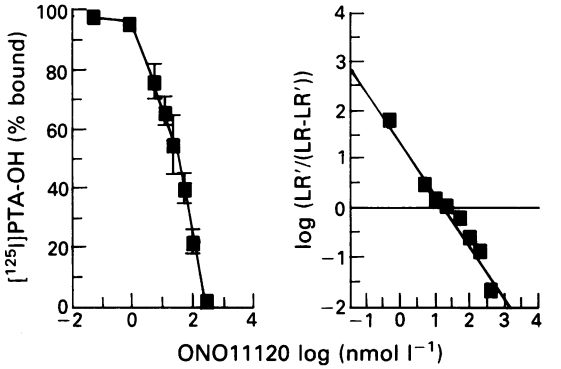

Figure 3 Hill plot of the displacement curve. K_i, calculated according to Cheng & Prusoff (1973), was 19 ± 4 nmol l^{-1} (n = 10, n Hill = 0.942).

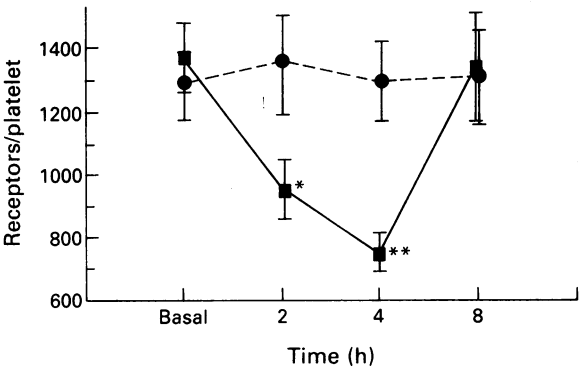

Figure 4 Number of TxA_2 receptors per platelet after placebo (●) or picotamide administration (■, 300 mg orally). *P < 0.05, **P < 0.01.

Discussion

Present results indicate that picotamide administration to humans induces a reduction of the TxA_2 binding sites available on the platelet membrane. This effect is reversible and is not observed 8 h after the last administration.

The kinetics of platelet TxA_2 receptor desensitization observed *in vivo* and the kinetic studies of [^{125}I]-PTA-OH

Table 1 TxA_2 receptor number and dissociation constant after picotamide or placebo administration (mean ± s.d.)

	Basal	*2 h*	*4 h*	*8 h*
Binding sites/platelet				
Picotamide	1366 ± 237	957 ± 221*	753 ± 119**	1362 ± 324
Placebo	1291 ± 211	1360 ± 324	1294 ± 258	1312 ± 308
Dissociation constant (nmol l^{-1})				
Picotamide	19 ± 3.8	17 ± 5.7	23 ± 7.8	22 ± 4.2
Placebo	21 ± 4.1	19 ± 2.9	21 ± 6.2	18 ± 3.8

* = $P < 0.05$, ** $P = < 0.01$

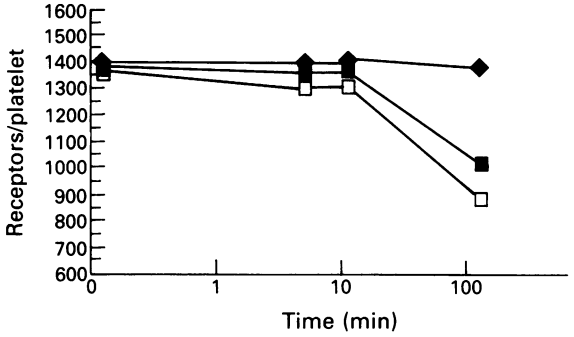

Figure 5 To evaluate the occupancy of the thromboxane platelet receptors by picotamide after platelet washing according to the protocol used, separate samples of platelet rich plasma (PRP) were incubated with ONO11120 (□, 1 μmol l^{-1}), picotamide (■ 2 μmol l^{-1}) or buffer (◆) at 37° C. Before and after 5, 10 and 120 min of incubation, samples were washed and binding sites for [^{125}I]-PTA-OH were determined (see **Methods**).

inhibition by picotamide seem to stand against the possibility that the reduction of specific [^{125}I]-PTA-OH binding sites could be simply due to the incomplete removal of the agonist with residual receptor occupation. In fact during the platelet washing procedure performed during binding studies, picotamide is easily removed from its specific receptors due to its relatively high dissociation constant (Modesti *et al.*, 1989). Moreover *in vitro* experiments confirmed that the TxA_2 binding capacity of platelets was unchanged after a short incubation with picotamide.

Desensitization of platelet thromboxane A_2 receptors by TxA_2 analogues has been recently reported to occur *in vitro* (Crouch & Lapetina, 1989; Liel *et al.*, 1988; Murray & Fitzgerald, 1989; Sato *et al.*, 1989). In fact platelets incubated with stable TxA_2 analogues rapidly (within few minutes) reach a desensitized state (Liel *et al.*, 1988; Murray & Fitzgerald, 1989). The sequence of events in TxA_2 receptor desensitization was found to involve initial uncoupling of the receptor from a guanine nucleotide binding (G) protein, followed by a receptor down regulation with a loss of specific membrane binding sites (Murray & Fitzgerald, 1989). In fact after an initial rapid uncoupling of the TxA_2 receptor and its associated G protein, a slow down regulation of receptors was observed. This pattern was observed to occur *in vitro* after incubation either with an agonist (U46619) (Liel *et al.*, 1988) or an antagonist (ONO11120) (Murray & Fitzgerald, 1989). The early desensitization after TxA_2 analogue stimulation could be physiologically relevant to the modulation of the platelet response to endogenous TxA_2. In fact TxA_2 potentiates platelet aggregation induced by other platelet stimulants (ADP, adrenaline) and is released by platelets in response to different stimuli or pathophysiological conditions, so that this desensitization could represent a regulatory constraint. On the other hand the late receptor down-regulation induced by TxA_2 analogues, which according to present data seems to occur *in vivo* too, could be relevant to the antiaggregating treatment with drugs interfering with TxA_2 platelet receptors. In fact this platelet response could potentiate the direct effect of the treatment.

The changes in the sensitivity of cells to an eicosanoid has also been studied in humans *in vivo* during the infusion of a PGI_2 stable analogue (iloprost) (Sinzinger *et al.*, 1981). The functional desensitization after iloprost infusion was found to be related to a reduction in the number of PGI_2 platelet receptors (Jaschonek *et al.*, 1988; Modesti *et al.*, 1987) and to a decrease in the amount of functional G_s (Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). The reduction of G_s also results in decreased responsiveness to other antiaggregating agents such as PGD_2 or adenosine A_2 which interact with G_s (Edwards *et al.*, 1987).

The mechanism underlying the receptor down-regulation is still under investigation, even though several hypotheses have been proposed such as internalization, proteolysis or phosphorylation (Hollemberg, 1985). A recent paper by our group (Modesti *et al.*, 1990), showed the internalization of ONO11120 after its binding to platelet specific TxA_2 receptors, so suggesting that the observed reduction may be due to the internalization of the receptor molecule and its consequent removal from the platelet surface.

In any case, the *in vivo* down regulation of TxA_2 binding sites even after the stimulation with a TxA_2 antagonist could represent an additional effect of the treatment, potentially enhancing the antiplatelet effect of TxA_2 antagonists.

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